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UNIVERSITY OF ALBERTA

# EVIDENCE FOR FREE RADICAL ACTIVITY ON TRAUMA-INDUCED SKELETAL MUSCLE IN THE RAT MODEL

BY

**JOAN M. MATTHEWS**

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHYSICAL EDUCATION AND SPORTS STUDIES

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Date: June 25, 1992

## DEDICATION

This degree is dedicated to my parents,

Jerry and Angela Matthews,

whose encouragement, support and love throughout the years enabled me to be where I am today. Thank you.

A special thanks is extended to Isabel Grondin who gave me the strength and encouragement that only a true friend could give.

**ABSTRACT**

Oxygen-derived free radicals (OFRs) have been implicated in a wide variety of pathological states such as exercise associated with sport, oxygen toxicity, inflammation and hypothermia. However, the precise role played by these individual reactive species and their products in normal and pathological process remains unclear. A free radical is defined as any substance possessing an unpaired electron in its outer orbit. The purpose of this research was to investigate the effects of endurance exercise, acute blunt trauma, and cold therapy on OFR activity in skeletal muscle. After an eight week training program, tissue malonaldehyde (MDA) and lipofuscin content in medial gastrocnemius (MG) and soleus (S) muscles were measured as indices of lipid peroxidation (reflecting possible OFR activity), and characterized modulations induced in OFR activity by trauma and treatment with cold. Following the common endurance training program, rats were randomly assigned to one of four groups in the study (E = exercise control; ET = exercise trauma; EC = exercise cold-treated; ETC = exercise trauma cold-treated). The endurance training program elicited significant ($p < 0.05$) increases in citrate synthase activity, but not indices of lipid peroxidation when compared to non-exercise controls. Following the acute blunt trauma to the hindlimb, indices of enhanced OFR activity were observed to increase slightly, but not to a significant degree. The administration of cold therapy prevented or delayed OFR

activity in both exercise MG muscle and exercise trauma MG muscle. Also, it was found that indices of lipid peroxidation varied with muscle type. There were significant differences ($p < 0.05$) in MDA and lipofuscin concentration between the MG muscle and the S muscle. These results demonstrated that cold therapy decreases lipofuscin cone in the traumatized MG muscle when compared to the corresponding trauma-control muscles. Lipofuscin cone was also decreased in the MG muscle when compared to the corresponding control muscles. From this study, it can be concluded that cold therapy significantly decreases indices of lipid peroxidation, suggesting a possible reduction in OFR activity in traumatized skeletal muscle.

Key Words:
oxygen free radicals, exercise, lipid peroxidation, cold, skeletal muscle, trauma, inflammation

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TABLE OF CONTENTS


| CHAPTER | | | PAGE |
|---|---|---|---|
| I | Introduction | | 1 |
| | 1.1 | Introduction | 1 |
| | 1.2 | Statement of Problem | 2 |
| | 1.3 | The Purpose of the Study | 3 |
| | 1.4 | Limitations of the Study | 5 |
| | 1.5 | Delimitations of the Study | 5 |
| | 1.6 | Definition of Terms | 6 |
| | 1.7 | Research Hypothesis | 8 |
| II | Review of the Literature | | 9 |
| | 2.1 | Introduction | 9 |
| | 2.2 | Oyxgen Free Radicals | 9 |
| | 2.3 | Exercise | 17 |
| | 2.4 | The Acute Inflammatory Response | 25 |
| | 2.5 | Cold Therapy | 34 |
| III | Methodology | | 39 |
| | 3.1 | Sample Selection | 39 |
| | 3.2 | Study Design | 39 |
| | 3.3 | Training Program | 39 |
| | 3.4 | Trauma-induced Injury | 40 |
| | 3.5 | Cold Treatment | 41 |
| | 3.6 | Procedures | 42 |
| | | 3.6.1 Citrate Synthase | 43 |
| | | 3.6.2 Oxygen Free Radical Detection | 44 |
| | | 3.6.2.a TBA-test | 44 |
| | | 3.6.2.b Fluorescence Intensity | 46 |
| | 3.7 | Statistics | 47 |
| IV | Results | | 49 |
| | 4.1 | The Effect of Exercise on Citrate Synthase Activity | 49 |
| | 4.2 | The Effect of Exercise and of Trauma on Indicators of Oxygen Free Radical Activity in Skeletal Muscle | 51 |
| | 4.3 | The Effect of Cold Therapy on Indicators of Oxygen Free Radical Activity in Skeletal Muscle | 51 |

V Discussion 57

VI Summary and Conclusion 63

6.1 Summary 63
6.2 Conclusions 64

Bibliography 66

Appendices

Appendix A: Experimental Design 78

Appendix B: Pilot Study 80

LIST OF TABLES


| | | PAGE |
|---|---|---|
| Table 1: | The endurance training program | 40 |
| Table 2: | The effect of exercise on citrate synthase enzyme in the medial gastrocnemius muscle | 50 |
| Table 3: | The effect of trauma and cold on indicators of oxygen free radical levels in the medial gastrocnemius muscle | 53 |
| Table 4: | The effect of trauma and cold on indicators of oxygen free radical levels in the soleus muscle | 54 |
| Table 5: | The effect of trauma and cold in the contralateral limb on indicators of oxygen free radical levels in the medial gastrocnemius muscle | 55 |
| Table 6: | The effect of trauma and cold in the contralateral limb on indicators of oxygen free radical levels in the soleus muscle | 56 |
| Table 7: | The effect of trauma on indicators of oxygen free radical levels in skeletal muscle (pilot study) | 81 |

LIST OF FIGURES


| | | PAGE |
|---|---|---|
| Figure 1: | A typical radical driven reaction: lipid peroxidation | 16 |
| Figure 2: | Experimental Design | 79 |
| Figure 3: | Experimental Design (pilot study) | 84 |

# CHAPTER 1

## 1.1 INTRODUCTION

The term "sports injury" is the result of the application to the body or part of the body of forces from athletic activities which exceed the body's ability to adjust to them (Teitz, 1989). Most injuries sustained in sport are essentially no different from those sustained in other activities, although the demands of the patient in terms of rehabilitation and return to activity may be significantly greater, and do require particular expertise and knowledge in their management.

In general, an athlete is a well-motivated and healthy person, however, an injury may cause a limitation or restriction on participation in that activity. Athletic injuries may result from a single traumatic episode or from repeated overuse. Virtually all physical activities and sports carry some risk of injury. An athlete's chance of sustaining some injury in a sporting event is approxiamately 50% (Arnheim, 1985). The most frequent injuries sustained by the active sports participant are muscle strains, joint sprains, contusions and abrasions. In fact, Arnheim (1985) states that 90% of sports injuries fall into this category.

The goal in managing these injuries is to identify these risks or damaging mechanisms, to employ methods and techniques to minimize them, and to implement adequate rehabilitation of

the musculoskeletal system for the early, and safe return of the athlete to participation.

## 1.2 STATEMENT OF PROBLEM

Acute soft tissue injury results in a cascade of vascular, cellular, and chemical events that lead to bleeding, inflammation, swelling, and pain, with loss of motion, joint stiffness and impaired motor function (Arnheim, 1985; Teitz, 1989). With acute injury, a localized tissue response is initiated and cellular changes occur. Cell injury and /or death results in the release of chemical mediators that increase cellular permeability and vasodilitation of capillaries. Soft tissue swelling results from fluid moving into the interstitial tissues. Swelling causes tissue hypoxia, which leads to cellular damage both directly and indirectly by increasing the release of chemical mediators. This change further increases cellular debris and a prolonged cellular response to injury. The end result is delayed tissue repair and healing (Leadbetter, 1990; Woolf, 1986).

The basis of this inflammation process is complicated and not fully understood. It should be studied as a foundation for initiating proper injury management. The overall goal of the treatment program is to implement a basic first aid to minimize the injury and to return the athletes to their desired level of participation as soon as possible. The steps of basic first aid are remembered by the acronym PRICES -

protection, rest, ice, compression, elevation and support of the injured part of the body (Arnheim, 1985; Teitz, 1989).

The application of cold in the first 24-72 hours during the initial inflammatory phase of injury is a well-accepted practice (Knight, 1985; McMaster, 1982; Meeusen, 1986). Although there is some confusion in the exact tissue response to cold, it remains popular in sports medicine as a means of injury management. Cold treatment decreases the acute reaction to injury by decreasing swelling, bleeding, pain, inflammation and muscle spasm. Cold slows cellular metabolism and impairs the inflammatory response (Barnes, 1979). Decreased bleeding and decreased swelling are probably secondary to vasoconstriction in response to cold (Clarke, 1958). Cold also decreases the firing of pain receptors which helps reduce muscle spasm (Thorsson, 1985). These effects of cold as a therapeutic modality appear to be accepted (Hocutt, 1981; Knight, 1985; Kowal, 1983; McMaster, 1982), although the mechanisms by which they occur have not been fully explained.

## 1.3 THE PURPOSE OF THE STUDY

The purpose of the present study was to investigate the activities of indicators of oxygen free radicals against the lipid peroxidation on skeletal muscle during the course of the acute inflammatory reaction with and without cold therapy, following a blunt trauma to the rat hindlimb.

In an attempt to find rational ways to reduce muscle

damage and help maintain cell viability, it is necessary to study the mechanisms by which loss of cell viability occurs following a variety of different stresses (McCord, 1985). Damage to skeletal muscle may occur in a number of different situations such as excessive or unaccustomed exercise, or acute soft tissue injury. Skeletal muscle, unlike other tissues, undergoes severe physical stresses and forces during exercise, and considerable insult in athletic activity.

Since the recognition that the oxygen-derived free radicals may be produced by biological reactions in skeletal muscle (Michaelis, 1949), the oxygen free radical has been implicated to play roles in exercise (Sexton, 1990), oxygen toxicity (McCord and Fridovich, 1978), phagocyte-mediated inflammation, and ischemia-induced tissue injury (McCord and Roy, 1982; McCord and Wong, 1980). An understanding of inflammatory cell stimulation after an acute injury may provide the basis for therapeutic intervention. Following an inflammatory reaction, if oxygen free radical activity propagates tissue damage, then inhibiting oxygen free radical production might reduce muscle damage. Regional hypothermia is known to protect many tissues resulting from oxygen free radical injury (Allen, 1938). If the use of cold therapy in the first aid phase of injury management is to decrease the acute inflammatory response to injury, it is therefore appropriate to examine the cellular responses to injury and the effect of a therapeutic modality, such as cryotherapy on

indicators of oxygen free radical activity.

Therefore, the purposes of this study were:

a) to determine if cold application affected oxygen free radical activity of trauma-induced skeletal muscle.

Sub-problems of this study were:

b) to determine if oxygen free radicals played a role in exercised skeletal muscle,

c) to determine if trauma-induced injury on skeletal muscle affected oxygen free radical levels.

## 1.4 LIMITATIONS OF THE STUDY

1. The accuracy and validity of the spectrophotometer and fluorometer,
2. The accurate measurement of the rat skin temperature during cold application,
3. The reproducibility of the trauma device.

## 1.5 DELIMITATIONS OF THE STUDY

The study was delimitated to:

1. Female rats,
2. Soleus and medial gastrocnemius muscles,
3. The application of cold treatment which was ten minutes, twice per day for three days, with the

cold pack stored at zero degrees celcius.

## 1.6 DEFINITION OF TERMS

Acute inflammation - the rapid onset reaction of the cellular and vascular elements of a tissue to an identifiifiable initiating injury caused by a trauma.

Cryotherapy - the therapeutic application of cold gel pack stored at -4 degrees celcius, applied to the skin surface which results in lowering the skin temperature.

Free radical - any atom, group of atoms or molecule in a particular state with one unpaired electron occupying an outer orbital.

Hypoxia - abnormally low partial oxygen pressures.

Inflammation - a localized tissue response initiated by injury.

Injury - the loss of cells and\or cellular matrix resulting from trauma (Teitz 1989).

Ischemia - local and temporary interruption of blood flow of the circulation to the hindlimb.

Lipid peroxidation - free radical attack on polyunsaturated fatty acids of the membrane.

Lipofuscin - a product of lipid peroxidation.

Malonaldehyde - a product of lipid peroxidation.

Phagocytosis - to ingest, neutralize and destroy the ingest particle.

Regeneration - a form of repair that produces new tissue that is structurally and functionally identical to normal tissue.

Repair - replacement of damaged or lost cells with new cells.

Sports-induced inflammation - inflammation resultant of an injury due to sports.

Trauma - an injury from an external force causing structural stress that results in a cellular or tissue response (Teitz 1989).

## 1.7 RESEARCH HYPOTHESIS

Three hypotheses were tested in the study:

1. There is no difference in the citrate synthase content between trained skeletal muscle and non-trained skeletal muscle.

2. There is no difference in indicators of oxygen free radical production in malonaldehyde (MDA) and/or lipofuscin between treatment groups and the control group in the medial gastrocnemius muscle.

3. There is no difference in indicators of oxygen free radical production in MDA and/or lipofuscin between treatment groups and the control group in the soleus muscle.

# CHAPTER 2

## REVIEW OF THE LITERATURE

### 2.1 INTRODUCTION

The purpose of this literature review is to outline and present data from the major areas of research that led to the undertaking of the present thesis. These areas will include: (a) basic concepts concerning oxygen free radical chemistry and their reactions in biochemical systems; (b) oxygen free radicals and their relationship to exercise; (c) the role of oxygen free radicals in inflammation; (d) and finally, oxygen free radicals in response to cold treatment.

### 2.2 OXYGEN FREE RADICALS

Presently, many researchers are interested in molecular oxygen and in excited oxygen states. That oxygen may be harmful to human health was first postulated with work on oxygen sickness (Lovlin, 1988), and finally caught the interest of biomedical scientists when McCord and Fridovich (1969) demonstrated that combining an oxygen radical known as superoxide with hydrogen produced hydrogen peroxide. It has now become apparent that not only are extreme concentrations of oxygen dangerous but even ambient oxygen may have both beneficial and adverse effects. Although the theory of oxygen toxicity is still being challenged, there has been continued evidence for its support (Fridovich, 1986). The potential

importance of free radical chemical events to sports medicine became evident when McCord (1974) demonstrated that an oxygen radical known as superoxide was involved in the inflammatory destruction of synovial fluid. More recently, McCord (1987) has advanced the reperfusion or reoxidation theory of tissue damage from an ischemic insult to interplay in the mechanisms of inflammation.

Free radicals by definition are molecules containing an unpaired electron in their outer orbit (Feher, 1987; Freeman, 1982; Pryor, 1973; Weiss, 1986). The electron orbitals of most chemical substances contain paired electrons with opposite spins. Because of the natural tendency to electron pairing, most molecules are stable. However, a molecule containing any unpaired electron results in an extremely reactive radical. When studying free radicals, one is constantly dealing with oxidation-reduction reactions. As first explained by Michaelis (1949), these reactions consist of an electron transfer from a donor (reducing agent) to a recipient (oxidizing agent). The loss of an electron is referred to as reduction whereas the gain of an electron is known as oxidation. The biomedical significance of the particularity of oxygen chemistry has been summarized by Gutteridge (1986) as follows: "The advantage of this process for aerobic life is a considerable slowing down of reactions of oxygen with nonradicals. The disadvantage however, is that one electron reduction of oxygen leads to the formation of

reactive oxygen species".

Radical formation is common. The chain of events that might occur in a typical radical driven reaction include three common features: (a) initiation, the production of the original active radical; (b) propagation, subsequent radicals form; and (c) termination, radicals react with other radicals or are scavenged to result in a stable form (Freeman, 1982; Chang, 1989; Weiss, 1986). Figure 1 illustrates this chain of events in a free radical reaction.

The area of free radical interest has centered on active oxygen species. The oxygen that exercise physiologists concern themselves with is known as ground state, molecular or dioxygen. Dioxygen is actually a diradical having two unpaired electrons in the outer orbitals. These two electrons are conceived of a spinning in the same direction. Based on conserving energy and time, dioxygen is said to "prefer" to receive one electron at a time which converts it into a superoxide radical. In the generation of some oxygen-derived free radicals, the sequence may propagate to form the potent hydroxyl radical. This extremely harmful hydroxyl radical is very reactive and will react with and alter the structure of whatever is nearby in the cell, and thus influences enzymes or cell membranes. Semiquinone radicals are the only non-protein carriers in the mitochondrial electron transport chain. They are embedded in the lipid phase of the membrane with a high mobility both across and along the membrane. Nohl (1986) has

provided evidence that the reaction produces the hydroxyl radical. Though radicals may be produced at a variety of points in the electron transport chain, Boveris (1976) has shown that when this pathway is active, the utilization of the semiquinone radicals usually provides the major source of the superoxide radical. Enzymes in the mitochondrial respiratory chain generate oxygen radicals even in the resting state (Chance, 1979). However, many of the compounds of the electron transport chain such as nicotinamide adenine dinusleotide (NAD), may react with oxygen to release the superoxide anion, and this may be considered the mitochondrial leak (Boveris, 1976; Del Maestro, 1980). Other sources of oxygen free radicals that this study is concerned with in addition to mitochondrial leaks, may arise from activated leukocytes (superoxide anion), and membrane-bound enzymes (xanthine oxide) of electron transport (Parks, 1988).

Although most studies have concentrated on the harmful effects of radicals, radicals play an important role in numerous physiological functions. A purposeful role of radicals is in the "respiratory burst" killing of pathogens by neutrophils and macrophages (Fantone, 1982). Some authors have speculated that superoxide may be essential to normal tissue function in phagocytes and is reduced beyond a critical level by oxygen free radical scavengers such as superoxide dismutase activities to prevent oxygen free radical-mediated damage (Del Maestro, 1980; McCord and Fridovich, 1969).

Patients unable to reduce molecular oxygen by radical reduction suffer from chronic granulomatous disease, causing recurrent infections (Del Maestro, 1980).

But, far more is known about the harmful consequences of free radicals. Free radical interactions have been implicated in processes which include inflammation, oxygen toxicity, and ischemia-induced tissue injury (McCord, 1987). In general, oxygen radicals and the reactive species which they generate alter the size and shape of compounds with which they interact with. For instance, oxygen-centered radicals can directly inactivate important enzymes, or may directly alter enzymes to initiate additional harmful reactions (Fridovich, 1986; Chance, 1979). The identification of oxygen-centered free radicals does not necessarily imply that their presence is responsible for toxic effects, but oxygen free radicals may induce toxic effects through the initiation of membrane lipid peroxidation (Freeman, 1982).

Enzyme activity may also be influenced indirectly through changes in membrane structure. In biological membranes, there is an important interaction between proteins and lipids, and essential to that interaction are polyunsaturated fatty acids (PUFA). Membranes are rich in PUFA which contribute to the membrane's fluid characteristic (Demopoulos, 1973a). However, PUFA are very susceptible to oxygen free radical attack. Lipid free radicals are also highly reactive and are capable of propagation and cell damage (Freeman, 1982). In Figure 1,

a radical abstracts a hydrogen ion and produces a radical which continues a chain radical reaction. When that process takes place in membranes, it is called lipid peroxidation. PUFA are especially susceptible to lipid peroxidation once it is initiated due to the abundance of univalent sites (Del Maestro, 1980; Miquel, 1989; Tappel, 1973). Lipid peroxidation may proceed in a controlled form without causing cell damage, but it may also lead to tissue damage, either reversible or irreversible. Tissue damage may be reversible if there is sufficient antioxidant and scavenger protection. If this is depleted, the damage becomes irreversible. Lipid peroxidation changes the fluidity of membranes (Taylor, 1983), and may cause membranes to become leaky resulting in a loss of, and an efflux of, enzymes which may induce damage (Halliwell, 1985a).

That such free radicals are constantly being formed, and that numerous other reactions are relevant involving them, and that they are not incidental side reactions is supported by the research into the effectiveness of antioxidants and radical scavengers in preventing and reducing tissue damage (McCord and Day, 1978; Tappel, 1973). Dean (1987) and Packer (1984) demonstrated that alpha-tocopherol (Vitamin E), embedded within the membrane, is a very important antioxidant in the defense against free radical membrane attack. In addition, there are numerous chain-breaking antioxidants such as cystine, glutathione, glucose, and vitamin C (Demopoulos,

1973b; Halliwell, 1985a). Also, there is a protective mechanism that has evolved in cells that are designed to prevent, limit or repair oxidative damage. These free radical scavengers which include superoxide dismutase, catalase and glutathione reductase (Brawn, 1980; McCord and Fridovich, 1969) are some of the oxygen free radicals that may terminate the reactions, unless the scavengers are overwhelmed with subsequent free radical activity.

Figure 1: A radical driven reaction: lipid peroxidation

Initiation: production of $HO^{\cdot}$

Propagation: $HO^{\cdot} + PUFA\text{-}H \longrightarrow LOO^{\cdot} \longrightarrow LOOH + L^{\cdot}$

Termination: $L^{\cdot} + TH_2 \longrightarrow LH + TH^{\cdot}$

$LOOH + TH_2 \longrightarrow LOOH + TH^{\cdot}$

Miquel (1989)

This sequence illustrates autoxidation of polyunsaturated fatty acids in lipid membranes:

$HO^{\cdot}$ = hydroxyl radical
PUFA = polyunsaturated fatty acid
$LOO^{\cdot}$ = lipid oxy radical
$L^{\cdot}$ = lipid radical
$TH_2$ = vitamin E
$TH^{\cdot}$ = vitamin E radical

Free radicals accidentally produced in the course of metabolism can therefore initiate a chain reaction of lipid peroxidation which will propagate along membranes. This process can result in several propagation steps before it is stopped by a termination reaction.

## 2.3 EXERCISE

The notion that exercise training induces adaptive responses that provide protection from various forms of injury and disease is accepted in the field of exercise science (Brooks, 1984; Fox, 1987). Exercise such as endurance training stresses various organ systems producing physiological responses. Training-induced adaptations will produce significant changes in the musculoskeletal system (Fox, 1987). Training provides the muscle with the ability to meet higher metabolic demands that otherwise would lead to decompensation. There are numerous changes in skeletal muscle that occur as a result of endurance training (Fox, 1987). In addition to increasing the oxidative capacity of skeletal muscle, exercise training may also alter the activity of one or more of the scavenging enzymes (Alessio, 1988; Higuchi, 1985; Ji, 1988a; Ji, 1988b; Laughlin, 1990).

Skeletal muscle has a large requirement for energy during exercise, such that there is a vast increase in mitochondrial activity and in oxygen consumption by the muscle (Brooks, 1984). The movement of electrons (free radicals by definition) is utilized as a means of energy transfer in the elctron transport chain. Here, oxgyen free radicals play an essential physiological role in normal muscle function (Del Masestro, 1980). Since many of the radicals proposed to have damaging effects on tissue are oxygen-derived, this increase in oxygen demand provides an increased initiator for these

radicals which may predispose the muscle to this type of damage. If oxygen is to be efficiently reduced by the muscle, it is a natural consequence that researchers would begin to question the role of exercise and the oxygen free radical.

Several workers have proposed that free radicals are produced in skeletal muscle during exercise. Gale (1974) has demonstrated an increased sensitivity to oxidizing agents which led to the possibility that free radicals might be generated in the muscle during exercise. This possibility was first suggested by Dillard (1978), who examined the effects of exercise and vitamin E in man on the levels of pentane excretion, formed as a lipid peroxidation product. This experiment was later repeated and confirmed in animals by Gee (1981) who found that during exercise at 50% of maximum oxygen uptake, there was a significant increase in breath pentane excretion, which was partially inhibited by oral vitamin E supplements. Other evidence for an increase in free radical activity during exercise has been presented by Brady (1979) who observed that malonaldehyde content, a byproduct of oxygen free radical reactions, in tissues in the rat increased following swimming exercise. Davies (1982) also found that the malonaldehyde content of muscle was increased in the rat following running exercise, and that a single bout of exercise could increase the free radical signals observed by electron paramagnetic resonance in rat skeletal muscle. Jackson (1985) demonstrated that certain free radical signals can be readily

and reproducibly detected in muscle tissue influenced by contractile activity, using relatively simple techniques.

The effect of endurance exercise training on oxygen free radical production may inhibit or accelerate the damage processes in skeletal muscle. First, muscular exercise commonly results in injury to the active muscles (Armstrong, 1984). The muscle injury resulting from exercise, referred to as exercise-induced injury, may be initiated through oxygen free radical formation (Armstrong, 1990; Duan, 1990). Secondly, exercise increases metabolism, and metabolic leaks are now thought to be an important source of oxygen-centered radicals (Jenkins, 1988). The mitochondria metabolize most of the molecular oxygen which implies that the mitochondria have a highly efficient antioxidant defense system. However, the production rate increases depending on the oxygen consumption, which may produce increases in the reactive oxygen species (Asayama, 1990). In the muscles of rats subjected to a program of strenuous exercise, Holloszy (1976) found the activities of the enzymes of the mitochondrial electron transport chain approximately doubled.

Skeletal muscle undergoes severe physical stresses and forces during exercise, which may induce the formation of free radical species. Once damage is initiated, production of increased amounts of lipid peroxides and increased free radical activity results (Zerba, 1990). Most of the studies in which elevated levels of peroxidation products have been

demonstrated in muscle following exercise have involved experiments in which animals were exercised to exhaustion (Brady, 1979) and in these circumstances, exercise-induced damage may have occurred.

Exercise-induced muscle damage has been documented in humans (Evans, 1986) and animals (Armstrong, 1983; Brady, 1979; Kuipers, 1983). Unaccustomed exercise is accompanied by injury to fibers in the active skeletal muscles, particularly when the exercise is relatively intense, is of long duration, and/or includes eccentric contractions (Duan, 1990). The mechanisms underlying exercise-induced muscle injury are not known. However, exhaustive exercise known to induce muscle damage has been shown to produce metabolic disturbances and increased free radical activity (Ebbeling, 1989). Kanter (1988) found a significant correlation between an increase in creatine kinase, an indicator of muscle damage, in the blood of runners in an 80 km race and an increase in indicators of oxygen free radical levels. Maughan (1989) measured reactive substances to oxygen free radicals in a variety of conditions following 45 minutes of downhill running which suggested a possible relationship between free radical generation and exercise-induced muscle damage. DeVries (1966) suggested that exercise may initiate a cycle in which local ischaemia led to muscle spasm, which in turn caused compression of blood vessels and additional ischaemia. The progressive increase in ischaemia could reduce oxygen availability to the muscle,

alter ion concentrations, produce ATP deficiency and increase waste accumulation. Ischemia-induced injury resembles cell damage following exhaustive endurance exercise, and among the factors that may influence the damage and repair processes are oxygen free radicals (Ebbeling, 1989; Hoppeler, 1986; Sjostrom, 1987).

Thus, there is data to suggest a relationship between free radical reactions and loss of membrane integrity as a result of muscle damage following exercise. However, the relationship may not be a causative one (Maughan, 1989). The source of the initiating events has not been identified, but several possibilities can be hypothesized. A cascade of events may occur that involves an initial mechanical injury, followed by the activation of macrophages and phagocytosis, a loss of structural integrity of cell membranes, a high concentration of calcium ions, and increased levels of some markers of lipid peroxidation (Jackson, 1988). Korthius (1985) and Laughlin (1990) suggested that xanthine oxidase represented the principle source of oxygen free radicals. By using a comptetitive inhibitor of xanthine oxidase, vascular permeability was prevented. In addition, mitochondrial electron transport chains have been identified as a potential source of free radicals (Ji, 1988a; Packer, 1984). Training is accompanied by an increase in mitochondrial activities and may therefore generate greater amounts of oxygen radicals then seen in mitochondria of untrained individuals (Boveris, 1973;

Fantone, 1985).

It is certain that exercise can induce muscle damage, but oxidant formation during exercise may be a consequence of muscle injury rather than a cause of it. It has been demonstrated that exercise represents a stress on animals (Gollnick, 1973; Vani, 1990). Oxidative stress is defined as the inability of the cell to defend itself against the oxygen-derived free radical species, resulting in oxidative injury (Halliwell, 1985a). Moreover, muscle adapts to the stress of repeated exercise such that subsequent bouts of exercise result in little or no damage (Brooks, 1984). As a result of exercise, skeletal muscle is in an oxidative stress situation due to the increase of toxic oxygen radicals that can elicit damage to cell membrane lipids and mitochondria. Protection against oxidative damage might be offered by careful endurance training. Adaptations of skeletal muscle mitochondrial enzymes have been proposed to be the main cause of the increased endurance capacity of trained individuals (Davies, 1981; Holloszy, 1973; Laughlin, 1990), and similarily, exercise may cause the antioxidant defense capacity to undergo adaptation (Asayama, 1990).

Cells are equipped with both nonenzymatic and enzymatic scavenging systems to eliminate oxygen-derived free radicals (Del Maestro, 1980). Distribution of these enzymes in various organs and tissues is related to the metabolic rate and production of oxygen radicals. High activities of superoxide

dismutase (SOD) and catalase are found in the liver, kidney, and erythrocytes, whereas skeletal muscle, which has a low resting metabolic rate, possesses relatively low antioxidant enzymes (Asayama, 1990). Antioxidant enzymes such as SOD, catalase and glutathione peroxidase (GPX) are present in aerobic cells and are essential to cells in removing superoxide and hydrogen peroxide from the tissues exposed to oxidative stress (Chance, 1979). As shown by Davies (1982) and Dillard (1978), the increased production of oxygen radicals and hydrogen peroxide during prolonged aerobic exercise can exceed the capacity of the antioxidant enzymes, thereby changing the intracellular redox state or causing oxidative damage. Antioxidant enzyme activities have been shown to change under certain conditions such as exercise. In exercise trained mice, different antioxidant enzymes showed increased activity (Kanter, 1988). In this animal study, damage suggested to be mediated by free radicals and lipid peroxidation was found to be significantly less. Davies (1981) suggested that the increased mitochondrial oxidative capacity in endurance trained rats may reduce the oxidative damage during exercise. Salminen (1988) reported that activities of catalase and GPX in mouse quadriceps muscle increased significantly a few days after a single bout of exhaustive running, indicating a possible adaptation of these two protective enzymes. This study was also the first to report that there was a significant difference in the rate of

peroxidation between white and red muscle fibres in mice. Quintanilha (1983) and Packer (1984) found that such training increased the activities of oxygen free radical scavengers in rat skeletal muscle. Jenkins (1984) showed that catalase activity was elevated in the trained state in human skeletal muscle. Ji (1988a) discovered that the adaptation of GPX increased significantly with endurance training in rat skeletal muscle, confirming that chronic exercise training could induce antioxidant enzymes in muscle. Experiments by Laughlin (1990) indicated that the activities of antioxidant enzymes in muscle paralleled the oxidative capacity of all the muscle samples investigated. These studies document an improved antioxidant status affording better protection against oxidative stress.

The foregoing studies clearly indicate that training not only enhances the capacity of the muscle to utilize oxygen, but also improves its ability to cope with the increased production of peroxides and free radicals during prolonged aerobic exercise. Thus, the antioxidant capacity acquired by exercise protects skeletal muscle against oxidative stress and lends support to the hypothesis that exercise training increases the resistance of the cell to oxygen free radicals, reducing the susceptibility of the muscle to peroxidative damage.

## 2.4 The Acute Inflammatory Response

Sports and physical exercise bring some risks of injury. An athlete's chance of sustaining some injury is approximately fifty percent (Arnheim, 1985). This localized tissue response to injury is known as inflammation, a defence reaction whose purpose is protection by preventing the spread of the injury, eliminating the insult, and removing the injured tissue (Movat, 1979; Rubin, 1988). It is a necessary process for healing and promotes regeneration (Leadbetter, 1990; Rubin, 1988). Inflammation acts as a signal of distress whenever biomechanical or metabolic limits are exceeded. It is a time-dependent evolving process characterized by vascular, chemical and cellular events (Kellert, 1986). Oxygen free radicals may be accountable for the damage to skeletal muscle cell membranes during the period of inflammation (Feher, 1987; McCord, 1987; Miquel, 1989). Disturbances that evolve during the inflammatory process may predispose skeletal muscle to the production of oxygen free radicals and their subsequent repercussions. By understanding the pathophysiology of sports-induced inflammation at the cellular level, ways to modify the repair process and potentially to allow an early return to activity may be revealed. The focus of this review will be the current evidence implicating the role of oxygen-derived free radicals (OFRs) and their metabolites in neutrophil- and macrophage-mediated tissue injury.

There is evidence in recent reports that during or after

acute blunt trauma in skeletal muscle, a series of events may occur that includes an initial mechanical injury, a severe breakdown of membrane phospholipids and an increased rate of protein degradation followed by edema formation (Newsholme, 1983; Fisher, 1990b). Moreover, an important activation of the acute inflammatory phase is demonstrated, which could partly account for membrane alterations (Hurley, 1983). In three models of induced inflammation in the rat model, McCord (1983) found that treatment with an oxygen radical scavenging enzyme, superoxide dismutase, suppressed the inflammatory response. Since the recognition that the superoxide free radical has been implicated to have a role in inflammation, if an inflamed tissue were infiltrated by active inflammatory cells, it would be vulnerable to radical-induced damage, such as lipid peroxidation and increase membrane alterations.

Immediately after an injury, an acute inflammatory response takes place in a short period of time where injured cells are disrupted or killed. This phase may last for up to 72 hours, depending on the severity of the injury, and constitutes a reaction involving both cellular and vascular elements (Leadbetter, 1990). The products of the inflammatory reaction include redness, swelling, pain, heat and loss of function (Hurley, 1983). The substances credited with these cardinal signs of inflammation are known as chemical mediators of inflammation (Rubin, 1988; Woolf, 1986). Tissue inflammation causes the release of these vasoactive substances

and chemical mediators that produce chemotactic factors, hemodynamic changes (vasodilation or vasoconstriction), increase in vascular permeability, and stimulate leukocyte migration (Rubin, 1988; Ryan, 1977).

Polymorphonuclear (PMN) leukocytes and mononucleated leukocytes are recruited to inflammatory sites as the result of a local generation of specific chemotactic factors (Leadbetter, 1990). The main function of the leukocytes, which are mainly comprised of neutrophils, monocytes, and macrophages is to provide the primary cellular defence via phagocytosis (Rubin, 1988). Phagocytosis is an active energy-dependent process, which is accompanied by a burst of metabolic activity (Hurley, 1983). Neutrophils, monocytes and macrophages enter inflammatory areas with the purpose of ingesting and disposing of unwanted material. Smith (1989) assessed the neutrophil infiltration following the reintroduction of oxygen after ischemia. The data suggested that the production of oxidant species occurred when the tissue neutrophil was dramatically increased. The conclusions drawn may be in part due to reactive oxygen metabolites generated by neutrophils, that after migration into the inflammatory area discharged OFRs (Fantone, 1982; McCord, 1987; Mehta, 1989).

During phagocytosis, the cell membrane engulfs the foreign agent and exposes the particle to degradative enzymes. In addition to releasing enzymes into the phagosome,

phagocytosis activates a membrane-associated nicotinamide adenine dinucleotide (NADPH) oxidase which oxidizes molecular oxygen to the superoxide anion free radical (Del Maestro, 1980; Weiss, 1989). The activation of this enzyme is associated with an increase in oxygen consumption known as the respiratory burst (Babior, 1973). In conjunction with an increase in oxygen consumption, neutrophils and macrophages have been shown to secrete both superoxide anion and hydrogen peroxide (Fantone, 1985; McCord, 1987). Recent studies have shown that most of the oxygen consumed by neutrophils after initiation of the respiratory burst can be accounted for by the superoxide secretion (Fantone, 1982; McCord and Roy, 1982). Babior (1978) indicated that the oxidase system of the neutrophil may be located within the lipid layer of the cell membrane since the cytoplasmic NADPH activates the enzyme system. Superoxide appears to be generated either within or on the external surface of the plasma membrane, accounting for the release of superoxide into the external environment after cell stimulation (Babior, 1978).

Leukocytes produce OFR secretions as a base function in muscle injury for bacterial killing (Fantone, 1982). The superoxide radical plays a key metabolic role in the phagocyte-mediated inflammatory response. If increased capillary permeability is advantageous for phagocytes to filter to an inflammatory area, then the tissue's ability to recognize OFRs, specifically the superoxide radical which

signals the presence of activated phagocytes, and to respond by lowering its permeability barriers is entirely appropriate (Fantone, 1982). This action enables the cells to expose the engulfed particles with a variety of oxidizing agents in addition to superoxide anion which includes hydrogen peroxide and the hydroxyl radical. Hydrogen peroxide may react with the metal catalyzed Haber-Weiss reaction which provides a mechanism for converting the relatively unreactive superoxide radical into the strongly oxidizing hydroxyl radical (McCord and Day, 1978). This mechanism appears to which account for the cytotoxicity of superoxide shown toward neutrophils (Salin, 1975).

The bactericidal process occurs within the phagolysomes thereby protecting the cell from the injurious effect of the radicals. Furthermore, this reaction is catalyzed by the scavenging enzyme superoxide dismutase. However, after an inflammatory reaction, normal scavenging systems are overloaded by an excessive production of OFRs and if greater than the tissue scavenger levels, tissue damage will occur, resulting in an increased permeability of cell membranes (Taylor, 1983). Del Maestro (1981) showed that the release of superoxide in the hamster cheek model resulted in an increased vascular permeability. Therefore, a significant proportion of the generated superoxide production is released into the extracellular space, which can result in additional reactive oxygen species and hydrogen peroxide formation

(Fantone, 1982; Del Maestro, 1980). The low concentration of scavengers present in the extracellular space results in the susceptibility to a superoxide anion damage (Salin, 1977; Babior, 1978). As a result, cell membranes may be the primary site of injury by PMN-derived oxygen metabolites. Also, superoxide anions and hydrogen peroxides are capable of permeating channels in the cell membrane and may initiate injury with intracellular molecules (Fantone, 1982; Movat, 1979).

An additional mechanism by which the superoxide radical in the inflammatory process may promote tissue injury is via the local generation of chemotactic factors (McCord and Roy, 1982). The radical serves to activate a latent chemotactic factor present in the extracellular fluids thereby enabling an activated neutrophil to create a chemoattractant, signalling its location to other nearby neutrophils. The superoxide-dependent chemotactic factor appears to play a role in the development of neutrophil-mediated inflammatory responses. The arriving neutrophil cells will in turn be stimulated to produce more chemoattractant, amplifying or maintaining the gradient until all stimulus is eliminated by phagocytosis (Taylor, 1983). A crucial characteristic to this proposed role of OFRs in the inflammatory process is that this chemoattractant has the ability to attract neutrophils without stimulating oxidative metabolism which prevents catalyzing its own activation (Del Maestro, 1980). Scavengers prevent the

activation of this chemoattractant although the concentration of scavengers in the extracellular fluids is low and may be overloaded after an inflammatory reaction (Babior, 1978).

The secretion of reactive oxygen metabolites and the release of enzymes may function to enhance tissue toxicity and degradation. An important mechanism by which reactive oxygen metabolites exert their toxic effect is the result of their ability to react with unsaturated lipids and initiate lipid peroxidation reactions in cell membranes, causing increased permeability of cell membranes (Korthius, 1985). If membranes undergo peroxidative change, the products of lipid peroxidation may be released into the extracellular space which may influence vascular permeability (Del Maestro, 1981) and leukocyte chemotaxis (McCord and Wong, 1980). Lipid peroxidation will initiate a strong inflammatory response with an extracellular release of superoxide radicals.

During an acute blunt trauma to skeletal muscle, metabolic events, such as partial or incomplete ischemia, results in altered ion concentrations, adenosine triphosphate (ATP) deficiency and waste accumulation have been proposed to explain muscle damage (Lee, 1987). Recent evidence indicates that injury observed after ischemia is mediated, in part, by reactive oxygen metabolites (Korthius, 1985; Walker, 1987). Ischemia results in a degradation of ATP and, hence to the triggering of the xanthine oxidase-mediated mechanism of tissue injury.

There is a large amount of circumstantial evidence which supports the proposal that xanthine oxidase is the source of oxygen radicals in ischemic skeletal muscle (Del Maestro, 1980; Korthius, 1985; McCord, 1987; Parks, 1988; Perry, 1987). Stripe (1969) showed that xanthine oxidase activity was present in skeletal muscle. It has been demonstrated that skeletal muscle is relatively resistant to ischemia (Barie, 1988; Labbe, 1987). ATP levels in muscle do not decrease appreciably during the first two to three hours of ischemia. However, studies by Perry (1987) and Bulkley (1987) suggest that partial ischemia, even for short periods of time can cause cell damage.

Following oxygen supply interruption, incomplete tissue ischemia results in ATP degradation to form hypoxanthine which produces the superoxide radical when xanthine oxidase is formed. Xanthine oxidase, which normally exists in the cell, is formed in response to low oxygen tension produced by ischemia. Accordingly, the addition of oxygen will trigger a burst of xanthine oxidase activity converting all accumulated hypoxanthine into superoxide, which may further undergo reduction to form hydrogen peroxide and the hydroxyl radical leading to an acute oxidative stress that may result in tissue damage (Labbe, 1987; Parks, 1988).

After prolonged ischemia, normal scavenging systems are overwhelmed by an excessive production of OFRs (McCord and Roy, 1982). Once again, superoxide can feed back to cause

neutrophil attraction into the damaged tissues, and the inflammatory process responds. Inflammation may initiate a cycle in which edema leads to the compression of the blood vessels, which reduces oxygen availability to the muscle, and ischemia is increased, adding insult to an initial transient ischemic injury (McCord, 1987).

These experiments provide insight into some of the mechanisms by which PMN-derived oxygen metabolites propagate and amplify the acute inflammatory response. The primary function of oxygen free radicals by activated inflammatory cells may be to aid in bacterial killing, however, peroxidative disruption of cellular membranes can also occur in the prescence of these same molecules. These findings suggest that OFRs enzymatically generated are associated with an increase in membrane permeability and an increase in leukocyte concentration. The permeability changes and resultant edema seen during the inflammatory response may be related to the extracellular release of superoxide and subsequent generation of activated oxygen species released from inflammatory cells.

## 2.5 Cold Therapy

The advantages of therapeutic cold in the rehabilitation of injury have been widely accepted (Arnheim, 1985; Knight, 1985; Kowal, 1983; McMaster, 1982). Several authors have reviewed the effects of cold on various physiological parameters, and because of these physiologic effects, the application of cold is beneficial in acute trauma (Clarke, 1958; Knight, 1985; Kowal, 1983; Shephard, 1983). In the treatment of a traumatic injury to the skeletal muscle, cold is used, both for its effect upon the response of the tissues from the trauma, and for the relief of pain.

The local reaction to injury is acute inflammation. The mechanism by which cold influences the events of the acute inflammatory process is inconclusive, but it is commonly accepted that cold diminishes the inflammatory response by decreasing the rate of tissue metabolism and reducing edema by promoting a vasoconstriction to counteract the vasodilation which follows soon after the injury (McMaster, 1982; Meussen, 1986; Thorsson, 1985). Cold also inhibits enzyme release and therefore there is less capillary breakdown than would normally be present after injury. Cold not only decreases the effects on the vascular membrane, it also decreases the effects of neutrohpil activiation (Moore, 1988), and leukocytes (Dorwart, 1973; McCord, 1974; Parks, 1988) which follow an inflammmatory reaction and which are potential sources of oxygen-free radicals in inflammation (Fantone,

1982; Fantone, 1985; Mehta, 1989; Weiss, 1989). Del Maestro (1981) proposed a relationship between the permeability changes and resultant edema seen during the inflammatory response to the extracellular release of superoxide radical and subsequent generation of activated oxygen species released from inflammatory cells. Furthermore, Korthius (1985) was able to block the expected increase in vascular permeability of skeletal muscle after an ischemic stress by using free radical scavengers. By blocking the free radical-mediated increase in capillary permeability, skeletal muscle was salvaged which otherwise would have become necrotic. With a hypothermic intervention, Dorwart (1973) reported that synovial fluid leukocytes exposed to cold had less phagocytosis and less inflammation than controls in the knee joints of dogs. Cold application appeared to limit the amount of tissue death and tissue debris by decreasing permeability (Dorwart, 1973). Similarily, McCord (1974) was able to protect synovial fluid against oxygen free radical degradation by phagocytizing polymorphonuclear leukocytes using free radical scavengers.

Furthermore, cold lowers the tissue temperature which decreases the metabolism and chemical actions of cells, and thus diminishes oxygen and nutrient needs in the affected cells and in the peripheral uninjured cells (Teitz, 1989). Cells are put in partial hibernation, and thus the extension of the injury is avoided. Otherwise, cells that survived the

initial trauma may not be able to withstand the lack of oxygen imposed by the disruption of local circulation. When a cell is deprived of oxygen, it switches to anaerobic metabolism (glycolysis) for its energy. As energy production decreases, membrane functions slow down, and the sodium pump's activity slows or stops, causing the sodium concentration within the cell to increase. As water is drawn into the cell, it swells and bursts. As well, intracellular acidosis further impairs membrane integrity causing enzyme release, a precursor to oxygen free radical generation and chemotaxis (Fantone, 1982). Together, the result leads to cell death, increasing the total amount of damaged tissue. Cold therapy can decrease or delay the amount of secondary hypoxic injury by decreasing tissue metabolism and the need for oxygen during the period of decreased oxygen delivery. In the event that the secondary injury is not minimized or delayed by cold, a hypoxic or ischemic environment develops.

Regional hypothermia has been shown to significantly increase the tolerance of extremities to ischemia (Allen, 1938). Allen (1938) was the first to note that regional hypothermia applied to an ischemic extremity reduced the limb loss and mortality rate and concluded that the lower the temperature, the longer the time that acute extremity ischemic could be safely tolerated. Duncan (1942) showed that the local application of ice also had a protective effect on an experimental extremity crush injury. It was found that

regional cooling of the extremity resulted in less local swelling if hypothermia was maintained during the compression period, but lowering of the temperature after the injury did not appear to affect the swelling. Paletta (1960) demonstrated that regional hypothermia significantly diminished edema in canine hindlimbs after five hours of ischemia. Wright (1988) demonstrated in the animal model that regional hypothermia was associated with a reduced size of skeletal muscle infarction in an ischemic injury of the canine gracilis muscle. These finding demonstrated a significant association between the regional distribution of vascular permeability, muscle injury, and temperature following an ischemic insult. A relationship is suggested, although the exact mechanism by which this relationship occurs is not clear from these studies.

Cold therapy and the inflammation process is not without problems as there are some contradictions. Brooks (1941) administered wounds via subcutaneous injections to the backs of rabbits. The inflammatory response was almost nonexistent in cold treated wounds, however after treatments were discontinued, it appeared that cold did not alter the inflammatory response, rather it just delayed the response. Abakumova (1978) also found the inflammatory response in wound healing in rats extended in time by hypothermia. Both the leukocytic and macrophagic phases occurred much later in hypothermic rats than under normal conditions. Others have

claimed that cold applications could significantly stimulate some types of inflammation by aggravating prostaglandin-mediated inflammation, while inhibiting other types such as those similar to traumatically-induced inflammation which are found to be inhibited by cold applications (Dorwart, 1973; Schmidt, 1979).

The control of skeletal muscle oxygen free radical injury might be achieved by the application of cold therapy. By preventing the accumulation of neutrophils and macrophages at the site of an acute inflammatory reaction, cold therapy may prevent tissue injury from the direct attack of superoxide radicals, and indirectly prevent tissue injury by all the additional mechanisms available by polymorphonuclear-leukocytes such as chemotaxis (Del Maestro, 1981; Walker, 1987).

# CHAPTER 3

# METHODOLOGY

## 3.1 Sample selection

All procedures described below were carried out in conformance with the guiding principles in the care and use of animals in the Canadian Council on Animal Care.

35 female Sprague Dawley rats weighing approximately 200-250 grams at the start of the experiment were housed in wire cages and provided with commercial rat chow and water ad libitum. At all times, the rats were maintained on a 12 hour light - 12 hour dark cycle.

## 3.2 Study Design

All animals (N=28) except for the sedentary control group (N=7) participated in the running program. At the end of the exercise program, rats were randomly assigned to one of four groups: exercise control, exercise trauma, exercise and trauma accompanied with cold treatment, and exercise with cold treatment (Appendix A).

## 3.3 Training Program

To accomplish the running program, a modified exercise protocol reported by Dudley (1982), Green (1983), Terjung (1976) and Williamson (1987) was used. The rats initially ran on a treadmill for 5 - 10 minutes, twice a day at 5 - 10

metres per minute, until they were accustomed to running, which occured within 3-5 days, as stated by Dudley (1982), using rats of a similar size as those used in this project. After the initial familiarization to running, the rats were progressively overloaded by gradually increasing speed and duration until 60 minutes of continuous endurance exercise was achieved.

The training program was employed for 8 weeks, 5 days per week, twice per day initially, and then at week 5, once per day. The program involved increases in both duration (minutes) and speed (m/sec). The speed of the treadmill was gradually increased to the desired setting over the first 2 minutes of the timed run. When the timed run was completed, the speed was brought to a gradual stop, with a 2 minute cool down. Table 1 indicates the training regime.

Table 1: The endurance training program.

| WEEK | DURATION (min) | GRADE(%) | SPEED (m/min) | SESSIONS PER DAY |
|---|---|---|---|---|
| 1 | 10 | 2 | 10 | 2 |
| 2 | 15 | 2 | 15 | 2 |
| 3 | 20 | 2 | 20 | 2 |
| 4 | 25 | 2 | 25 | 2 |
| 5 | 50 | 2 | 20 | 1 |
| 6 | 60 | 2 | 23 | 1 |
| 7 | 60 | 2 | 25 | 1 |
| 8 | 45 | 2 | 25 | 1 |

### 3.4 Trauma-induced injury

The rats to be traumatized were subjected to a single impact trauma to the medial aspect of the gastrocnemius muscle

of the left leg, using a humane and reproducable method reported by Stratton (1984), and modified by Fisher (1990b). The trauma model was designed for small rodents in which all traumatized muscles were exposed to similar forces. During this procedure, rats were briefly anesthetized with halothane and the hindlimb was shaved of fur. The crush-type injury was delivered once by dropping a weighted (700 g) solid aluminum cylinder with a flat impact surface, which had a surface area of 1.5 square cm. To ensure stabilization and accurate delivery, the cylinder was dropped 125 mm down a tubular guide fixed to a ring stand. The limb was positioned manually with the foot stabilized. The location of the large belly of the gastrocnemius muscle was determined by palpation, and the device was placed over its broadest region, with the tibia out of the line of the device to avoid fracture. The instantaneous force delivered by the impact device was 0.57 N.m. per square cm.

### 3.5 Cold treatment

The animals to receive cold treatment were categorized into two groups: exercise cold treatment, and exercise cold treatment following trauma. A three inch x two inch flex versal cold pack was used for the cold applications. There were six treatment times, and each cold treatment had an application time for 10 minutes, twice per day, once in the morning and once in the late afternoon for a 72 hour period at

an approximate skin surface temperature of 0 degrees celcius (Knight, 1985). The first cold treatment began immediately post-trauma in the exercise trauma group. The application of cold was to the medial aspect of the hindlimb of the rat, and the area treated was shaved of fur. The foot of the rat was maintained in extension by gentle traction on the distal phalanges as treatment was applied with the therapeutic cold pack. The other leg of the treatment animal was positioned away from the cold pack to minimize the likelihood that the leg may be affected. Rat skin temperature was determined prior to and throughout the experiment by a 4 channel Yellow Spring Temperature probe. The probe was placed between the cold pack and treatment area. The temperature of the probe placed between the cold pack and the skin surface was used as an estimate of the skin surface temperature, and any temperature falling below 0 degrees celcius resulted in the removal of the cold pack until rewarming occurred (Olsen, 1972).

### 3.6 Procedures

The rats were anesthetized while the muscle samples were excised, and then sacrificed. The muscles removed were the soleus and medial gastrocnemius muscles of the left hindlimb, and in trauma groups, the same contralateral muscles were collected. The muscles removed were immediately frozen in liquid nitrogen, and then stored at -70 degrees celcius until

lyopholized. Lyopholized tissues were then stored at -70 degrees celcius until analyzed in duplicate as follows: citrate synthase (CS), malonaldehyde (MDA), and lipofuscin. To ensure uniformity, precisely the same procedure was employed in each instance and the tissues were stored under the same conditions.

### 3.6.1 Citrate Synthase (E.C. 4.1.3.7)

The biochemical assay used to indicate an endurance training adaptation was the citrate synthase (CS) enzyme. This enzyme includes a marker from the metabolic pathway within the cell. CS is a Kreb cycle enzyme which was used to represent the state of cellular respiration (Lowry, 1978). The CS assay is a widely reported assay indicating enhanced enzyme activity (Terjung, 1976, Green, 1983).

The assay was prepared as follows (Srere, 1969): Lyopholyzed muscle tissue samples (.01 g) from the medial gastronemius muscle of the hindlimb of the rat were used. The tissue samples were homogenized (30x dilution) in 50 mM Tris-HCl buffer containing 1 mM EDTA and 0.1% Triton X-100, pH 7.6. Homogenate samples were then sonicated (Biosonik, Bronwill Scientific, N.Y., U.S.A.) 3 times for 5 seconds at 30 second intervals at 25% of maximal probe intensity to ensure the membrane disruption necessary for the enzyme analysis. Homogenate samples were then centrifuged (1000x g for 10 minutes, 4 degrees celcius) and the supernatant collected for

the analysis of CS enzyme. Five ul of tissue supernatant was added to a 590 ul stock solution of 0.1 mM DTNB, 0.35 mM acetyl CoA, and 50 mM Tris-HCl buffer (pH 8.1) twenty seconds before starting the reaction with the addition of 5 ul of 0.083 mM of oxaloacetate. Spectrophotometric analysis was performed at 412 nm, and linear rates were obtained for at least 2 minutes.

### 3.6.2 Oxygen Free Radical Detection

The reaction of free radicals with membrane lipids produces many biproducts which are often measured as a means of assessing lipid peroxidation (Halliwell, 1985b; Tappel, 1973). To obtain an indication of the degree of lipid oxidation of tissues, two independent methods were used in this study: the chemical-thiobarbituric acid (TBA) test and the fluorescence intensity test.

#### 3.6.2.a TBA-test

The TBA technique has been utilized by many authors (Bidlack, 1973; Buege, 1978; Dillard, 1971a; Kosugi, 1989; Lovlin, 1988) as a marker of lipid peroxdiation and an indirect indicator of free radical activity. Although measurement of this technique has been criticized (Gutteridge, 1981), most authors agree that it is a very useful marker for thiobarbituric reactive substances (TBARS) such as malonaldehyde (MDA) in membrane damage of tissues (Chang,

1989; Freeman, 1982; Halliwell, 1985b; Koller, 1987; Lovlin, 1988). The test for the detection of MDA is based on the reaction of MDA, that is generated from peroxidized samples, with TBA to give a pink chromophore (Kosugi, 1989).

The level of MDA in tissues was determined using the method reported by Buege and Aust (1978) and modified by Lovlin (1988). The HCL-thiobarbituric acid (TBA) reagent solution used to determine MDA was prepared as follows: 2.5 ml of concentrated HCL was added carefully to 97.5 ml of distilled water and mixed gently using a magnetic stirrer. Next, 0.375 ml was removed from the beaker and 0.375 g of TBA acid (powder) added to it making 100 ml of TBA reagent solution. To ensure dissolution of TBA powder, the beaker containing the solution was warmed to 37 degrees C on a temperature controlled magnetic stirrer. MDA was determined in duplicate as follows: 0.007 grams of the lyophilized tissues was added to 1 ml of distilled water in a test tube before addition of the HCL-TBA reagent solution. For each determination, two ml of the TBA reagent was added to the sample, mixed on a vortex-type mixing device and then digested for 8 minutes at 95 degrees C (boiling) in a water bath. At the end of the digestion period the samples were transferred to a second water bath at room temperature to cool. After cooling, 3 ml of butanol was added to the sample, the tube capped, and shaken vigorously for 5 seconds. After centrifugation at 3000 rpm for 10 minutes, an aliquot of the

butanol extract was transferred to a cuvette and the optical density at 532 nm (the maximum absorption for MDA) determined with a spectrophotometer. A stock solution of malonaldehyde was used to form a series of dilutions over the range 250 nM to 1000 nM. The mean of 2 determinations per sample was recorded and used in the statistical analyses.

3.6.2.b Fluorescence Intensity Test

Other products are formed when lipids are oxidized. More recently, it has been shown that MDA reacts with amines to form yellow fluorescent chromophores (Chio, 1969a; Chio, 1969b; Dillard, 1971b), and the intensity of the fluorescence was directly related to the degree of peroxidate damage (Dillard, 1971a). Dillard (1971a) concluded that an advantage to using the fluorescence method was the high sensitivity of the technique. The fluorescent characteristics of products formed during lipid peroxidation were studied previously (Chio, 1969a), and the production of these products was shown to be a function of time of peroxidation. Lipofuscin, a lipo pigment, is a product of lipid peroxidation, and thus its prescence is evidence for lipid peroxidation occuring (Tappel, 1973). Lipofuscin was estimated by a modificatin of the fluorescent method of Bidlack and Tappel (1973) who found that the development of fluorescence was related to formation of TBARS during lipid peroxidation.

The assay was prepared as follows: 5 mg of lyopholized

tissue were added to 4 ml of an extraction mixture consisting of 2:1 (v:v) chloroform:methanol. To prevent further oxidation of lipids in the samples or evaporation of the mixture, the samples were capped. Each sample was then vortexed for 2 minutes, concentrating the lipofuscin in the chloroform. A further 2 ml of distilled water was added to the test tube, and the contents mixed for 5 seconds. The mixture was then separated into two phases by centrifigation (1000x g for 10 minutes) and the chloroform layer was examined for fluorescence using a fluorometer (BeckTurner model #110). The following settings were used: The filters for excitation was 365 nm and emission measured at 435 nm. The meter multiplier was set at 7x. The slit arrangement (1,3,10,30) was set at 10 fold. The lamp was set between 300-500 nm, which peaked at approximately 360 nm. With these instrument parameters, results were expressed in relative fluorescence intensity units using tetraphenylbutadiene (0.0000001 M) as a reference standard. The flourescence intensity of 2 standard series of dilutions formed a straight line.

## 3.7 Statistics

Tissue analysis was completed in duplicate to increase the reliability of the values. Tissue MDA and lipofuscin contents between groups were compared by a one-way analysis of variance with the Newman-Keuls post hoc test for differences.

Tissue MDA and lipofuscin contents of the injured and uninjured limbs of the two traumatized groups (exercise trauma, and exercise trauma cold-treated groups) were compared by a two-way anova with the Newman-Keuls post hoc test for differences. Citrate synthase contents between control and exercise groups were compared by an unpaired t-test analysis. The $p < 0.05$ was accepted as being statistically significant.

# CHAPTER 4

## RESULTS

### 4.1 The Effect of Exercise on Citrate Synthase Activity

A statistical analysis of the effect of exercise on citrate synthase in the medial gastrocnemius muscle was undertaken (Table 2). Analysis of the training effect in the medial gastrocnemius muscle indicated that the training program induced a significant increase ($p < 0.05$) in the citrate synthase activity when compared to the sedentary control group. Although these results for the medial gastrocnemius muscle represent only what has occurred in mixed muscle and not the response of any given skeletal muscle fiber type, exercise training of the endurance type induces an increase in the oxidative capacity of all three fiber types (Green, 1983; Holloszy, 1973; Terjung, 1976). Therefore, it can be assumed that the two muscles (medial gastrocnemius and soleus muscles) in the four training groups, underwent changes indicative of a training effect.

Table 2: Effect of Exercise on Citrate Synthase Enzyme in the Medial Gastrocnemius Muscle

| | Citrate Synthase umoles / min . g |
|---|---|
| Sedentary Control | 8.1 |
| Exercise Control | 27.7 |

Values are significantly different ($p < 0.05$) for independent groups in a Student's T-test between the control and the exercise groups ($n = 6$).

Rats were exposed to an exercise program (exercise control) or were not exercised (control). Observations of citrate synthase enzyme were made at the end of the exercise program as outlined in Chapter III.

## 4.2 The Effect of Exercise and of Trauma on Indicators of Oxygen Free Radical Activity in Skeletal Muscle

The effect of exercise and the effect of trauma on malonaldehyde and lipofuscin levels of medial gastrocnemius and soleus muscles were determined (Table 3 and Table 4 respectively).

As shown, the activities of malonaldehyde and lipofuscin in the exercise control group and in the exercise trauma group did not result in any significant differences ($p > 0.05$) when compared to the sedentary control or the exercise control group in either the medial gastrocnemius or the soleus muscles.

## 4.3 The Effect of Cold Therapy on Indicators of Oxygen Free Radical Activity in Skeletal Muscle

The effect of cold on malonaldehyde (MDA) and lipofuscin levels of medial gastrocnemius and soleus muscles were determined (Table 3 and Table 4 respectively).

After three days of intermittent cold treatment applied to the gastrocnemius muscle, the rats exposed to cold treatment showed significant decreases ($p < 0.05$) in lipofuscin compared to the groups not treated with cold. Post hoc tests for multiple comparisons showed that the exercise

cold-treated group and the exercise injured cold-treated group differed significantly ($p < 0.05$) from the sedentary control, the exercise control, and the exercise injured group in the lipfuscin assay. The only significant difference ($p < 0.05$) found in MDA analysis in the medial gastrocnemius muscle was the effect of trauma on exercised muscle which showed a significant increase when compared to the effect of cold on exercised muscle.

As determined in Table 5, the contralateral medial gastrocnemius muscle which did not undergo direct trauma was not significantly different ($p > 0.05$) in either the exercise injured rat or the exercise injured cold-treated rat. Table 6 determined significant differences ($p < 0.05$) in the soleus muscle were found only in MDA content between the exercise injured limb and the exercise injured cold limb, from their respective untreated counterparts. The contralateral muscle of the exercise injured group and the exercise injured cold-treated group had a significantly lower ($p < 0.05$) MDA content. Lipofuscin levels between treatment groups in the soleus muscle were not significant ($p > 0.05$).

Table 3: Effect of Trauma and Cold on Indicators of Oxygen Free Radical Levels in the Medial Gastrocnemius Muscle

| | ** Malonaldehyde umoles / mg | * Lipofuscin nmoles / mg |
|---|---|---|
| Sedentary Control | 0.108 | 0.0158 [a] |
| Exercise Control | 0.122 | 0.0141 [a] |
| Exercise Cold | 0.131 [a] | 0.0087 [b] |
| Exercise Injured Rat | 0.084 [b] | 0.0186 [a] |
| Exercise Injured Cold Rat | 0.110 | 0.0086 [b] |

a,b
Values with identical alphabetical superscripts are significantly different ($p < 0.05$) versus other groups in the same column ($n = 7$).

* expressed relative to butadiene fluorescence ($10^{-7}$ mol).

** expressed relative to tetraphynelbutadiene ($10^{-7}$ mol).

Rats were exposed to an exercise program (exercise control) or were not exercised (control). Rats were given a single impact trauma to the medial aspect of the left hindlimb (injured) or were not injured (controls). In injured rats, both the injured leg and uninjured leg were studied. Rats were given cold treatments (cold group and injured-cold group) twice daily starting on day 1 (immediately post-trauma) and continued intermittently until day 3 (0 degrees Celcius x 10 min for 3 days) to the medial aspect of the hindlimb. Observations of MDA and lipofuscin levels were determined 72 hours post-treatment.

Table 4: Effect of Trauma and Cold on Indicators of Oxygen Free Radical Levels in the Soleus Muscle

| | ** Malonaldehyde umoles / mg | * Lipofuscin nmoles / mg |
|---|---|---|
| Sedentary Control | 0.242 | 0.0218 |
| Exercise Control | 0.267 | 0.0245 |
| Exercise Cold | 0.225 | 0.0158 |
| Exercise Injured Rat | 0.244 | 0.0245 |
| Exercise Injured Cold Rat | 0.245 | 0.0225 |

No significant differences were determined (n = 7).

* expressed relative to butadiene fluorescence ($10^{-7}$ mol).

** expressed relative to tetraphynelbutadiene ($10^{-7}$ mol).

Rats were exposed to an exercise program (exercise control) or were not exercised (control). Rats were given a single impact trauma to the medial aspect of the left hindlimb (injured) or were not injured (controls). In injured rats, both the injured leg and uninjured leg were studied. Rats were given cold treatments (cold group and injured-cold group) twice daily starting on day 1 (immediately post-trauma) and continued intermittently until day 3 (0 degrees Celcius x 10 min for 3 days) to the medial aspect of the hindlimb. Observations of MDA and lipofuscin activity were determined 72 hours post-treatment.

Table 5: Effect of Trauma and Cold in the Contralateral Limb on Indicators of Oxygen Free Radical Levels in the Medial Gastrocnemius Muscle

| | ** Malonaldehyde umoles / mg | * Lipofuscin nmoles / mg |
|---|---|---|
| Exercise Injured Rat | | |
| Injured limb | 0.084 | 0.0186[a] |
| Uninjured limb | 0.091 | 0.0193 |
| Exercise Injured Cold Rat | | |
| Injured limb | 0.110 | 0.0086[a] |
| Uninjured limb | 0.124 | 0.0123 |

[a] Values with the alphabetical superscript are significantly different ($p < 0.05$) versus other groups in the same column ($n = 7$).

* expressed relative to butadiene fluorescence ($10^{-7}$ mol).

** expressed relative to tetraphynelbutadiene ($10^{-7}$ mol).

Rats were exposed to an exercise program (exercise control) or were not exercised (control). Rats were given a single impact trauma to the medial aspect of the left hindlimb (injured) or were not injured (controls). In injured rats, both the injured leg and uninjured leg were studied. Rats were given cold treatments (cold group and injured-cold group) twice daily starting on day 1 (immediately post-trauma) and continued intermittently until day 3 (0 degrees Celcius x 10 min for 3 days) to the medial aspect of the hindlimb. Observations of MDA and lipofuscin levels were determined 72 hours post-treatment.

Table 6: Effect of Trauma and Cold in the Contralateral Limb on Indicators of Oxygen Free Radical Levels in the Soleus Muscle

| | Malonaldehyde** umoles / mg | Lipofuscin* nmoles / mg |
|---|---|---|
| Exercise Injured Rat | | |
| Injured limb | 0.244[a] | 0.0245 |
| Uninjured limb | 0.186[a] | 0.0293 |
| Exercise Injured Cold Rat | | |
| Injured limb | 0.245[b] | 0.0225 |
| Uninjured limb | 0.190[b] | 0.0228 |

a,b Values with identical alphabetical superscripts in the same column are significantly ($p < 0.05$) different ($n = 7$).

* expressed relative to butadiene fluorescence ($10^{-7}$ mol).

** expressed relative to tetraphynelbutadiene ($10^{-7}$ mol).

Rats were exposed to an exercise program (exercise control) or were not exercised (control). Rats were given a single impact trauma to the medial aspect of the left hindlimb (injured) or were not injured (controls). In injured rats, both the injured leg and uninjured leg were studied. Rats were given cold treatments (cold group and injured-cold group) twice daily starting on day 1 (immediately post-trauma) and continued intermittently until day 3 (0 degrees Celcius x 10 min for 3 days) to the medial aspect of the hindlimb. Observations of MDA and lipofuscin activity were determined 72 hours post-treatment.

# CHAPTER 5

## DISCUSSION

The study was undertaken to determine the effects of therapeutic cold treatment on exercised skeletal muscle (medial gastrocnemius and soleus muscles) following acute blunt trauma. The study was also undertaken to determine the effect of endurance training on indicators of oxygen free radical activity and the effect of trauma on indicators of oxygen free radical activity in the medial gastrocnemius and soleus muscles.

Following the endurance training program, there was a significant increase ($p < 0.05$) in the activity of citrate synthase in the medial gastrocnemius muscle. The citrate synthase activity of the medial gastrocnemius muscle of rats in the exercise group was nearly four times that of the control group. The exercise-training program used in this study produced increases in the oxidative capacity of the skeletal muscles of the exercised rats. This study achieved results in the same direction as Green (1983) and Terjung (1976), who demonstrated an increase in citrate synthase activity using a similar protocol.

However, the exercised muscles showed no changes in MDA or lipofuscin levels in either the medial gastrocnemius or soleus muscles when compared to the control muscles. It is possible that training-induced alterations in skeletal muscle oxidant scavenger enzymes account, at least in part, for the

apparent beneficial effects of endurance training. This lack of significant differences may indicate that daily endurance exercise training could induce adaptations such as an increase in oxidative enzymes within the muscle, resulting in an increased resistance to oxygen free radical-mediated damage (Alessio, 1988; Asayama, 1990; Ji, 1988a; Ji, 1988b; Laughlin, 1990).

Trauma had variable effects on MDA and lipofuscin levels in the muscles studied. Although not to a significant degree ($p > 0.05$), trauma increased the lipofuscin level in injured medial gastrocnemius, decreased the MDA level in injured medial gastrocnemius, and did not affect MDA or lipofuscin levels in the soleus muscle. TBA-reactive material levels, used as a marker of lipid peroxidation, were lower than the values expected in the post-injury state. The TBA test is a widely used method for the study of lipid peroxides formed. Nevertheless, this test is based on a function of oxidative time since MDA and lipofuscin are products of a series of several free radical intermediate steps (Averet, 1990; Tappel, 1973). Muscles were examined at one interval post-injury. Therefore, this time-dependent experiment may have led to an undervalue of lipid peroxidation intensity. The values of MDA content and fluorescence intensity showed an increase as a function of oxidation time, 72 hours post-injury. The data suggest that oxygen-derived metabolites may play a role in tissue injury during an earlier period of macrophage

activation. Also, the number of oxygen free radicals produced by neutrophils and macrophages could vary with the experimental conditions and the extent and severity of injury produced by the trauma device (Fantone, 1982).

An additional pilot study was conducted to examine the immediate effect of trauma on indicators of oxygen free radical activity (Appendix B). MDA and lipofuscin production were observed in the medial gastrocnemius and soleus muscles during a control period, immediately post-trauma, and 5 minutes, 10 minutes, 20 minutes, 30 minutes, and 60 minutes post-trauma. Both MDA and lipofuscin levels were extremely high immediately post-trauma, and then returned to control levels within a five minute interval in both muscles. Levels gradually, but not significantly, increased during the following 60 minutes. The results of this study were inconclusive due to the large standard error (minimal n). However, there was a significant increase in oxygen free radical indicators in the muscles which received trauma when compared to non-traumatized muscles. The initial mechanical injury from the trauma may have produced the immediate significant levels, reflecting possible increases in oxygen free radical activity. The reactive hyperemia, which follows acute blunt trauma, may provide a beneficial effect by washing out potentially harmful substances capable of generating oxygen free radicals. Hence, the levels in the traumatized muscles return to control levels in a short time after trauma

(Lewis, 1983).

The contralateral limb of injured rats, which did not undergo direct trauma, was also studied as a control for possible systemic effects of trauma. The medial gastrocnemius muscle in the exercise trauma group and the exercise trauma cold-treated group did not show significant differences ($p > 0.05$) between the injured limb and the contralateral uninjured limb of the traumatized rat. Trauma produced a systemic effect. However, in the soleus muscle, both injured limb versus the contralateral uninjured limb in the exercise trauma group and in the exercise trauma cold-treated group did show significant differences ($p < 0.05$), indicating that it was the effect of trauma and/or of cold that produced the significant differences.

Oxygen free radical production during the acute inflammatory response appeared to respond to cold treatment. In this study, cold produced a significant reduction ($p < 0.05$) in the production of indicators of oxygen free radical activity. The explanation of the effects of cold on indicators of oxygen free radical activity seen in this study remains a matter for speculation. Many factors might influence the muscle response to cold such as the effect of exercise and/or the severity of the trauma. The cold treatment protocol could also vary according to the dose, temperature, and the time of application relative to the injury. By examining two muscles, the direct effect of trauma

and of cold was determined in the medial gastrocnemius muscle, and the soleus muscle illustrated the relative depth of tissue penetration of the trauma and the cold treatment, and how these affected oxygen free radical activity. Also, these factors may explain the differences found among the treatment groups.

From this study, tissue injury was in all likelihood the combined result of trauma, release of lysosomal enzymes, disruption of blood supply, and oxygen free radicals derived from phagocytic cells. Histamine, a well-known inflammatory mediator, increases skeletal muscle vascular permeability, but Korthius (1985) has suggested that histamine is not responsible for the increased vascular permeability induced by ischemic injury. He found that oxygen free radical scavengers significantly affected the vascular permeability. On the basis of this information, interventions can be used to intercept oxygen free radicals in a manner that protects the tissue from injury after the production of oxygen free radicals. Cold may inhibit neutrophil chemotaxis and the tissue infiltration of leukocytes, thereby decrease the extent of skeletal muscle necrosis (Walker, 1987). By decreasing the activation of inflammatory cells and by inhibiting enzymes that generate free radicals, cold treatment may be effective in protecting tissue against damage from oxygen free radicals. Therefore, secondary injury to surrounding cells, and capillary permeability induced by free radicals also are

reduced. The present results would appear to confirm those of other authors who have reported that cold is effective in decreasing lipid peroxidation.

Cold therapy has been widely recommended for the reduction of post-traumatic swelling, but in this study, cold application caused an increase in the tissue water content of injured and uninjured limbs, particularly in the subcutaneous tissue. These tissue results were interesting in that, even though the cold treated limbs were more swollen, the indices of lipid peroxidation were significantly reduced, indicating a decrease or delay in oxygen free radical production. These finding concur with Farry (1980) who concluded that this unwanted swelling could be minimized by combining cold treatment with ice and with compression and elevation. The reported edema is consistent with similar findings reported by Matsen (1975), who agreed with the hypothesis that the vessels in the superficial tissues are damaged by cold-induced ischaemia, and when the cooling is discontinued, the reperfusion of these damaged vessels increases edema. This is more in keeping with the beneficial clinical effects of ice therapy.

# CHAPTER 6

## SUMMARY AND CONCLUSIONS

### 6.1 Summary

In summary, it is apparent that the association between oxygen free radicals and the pathology in skeletal muscle can be explained if it is accepted that lipid peroxidation plays a role in a general mechanism of oxygen free radical muscle damage. It is important to stress that lipid peroxidation is an active role in the process of damage and that increased lipid peroxidation occurs prior to the loss of cell viability. The data presented does not prove that the oxygen free radicals were the cause or a consequence of the damage due to the acute blunt trauma but, it has previously been suggested that inflammation is associated with an increase in oxygen free radical-mediated damage to tissue (Del Maestro, 1981). The processes of inflammation are basically defensive in nature, however, inflammation also forms the basis for a series of potentially harmful effects to the damaged tissue. Substances which inhibit inflammation and lipid peroxidation might therefore reduce muscle damage. Cold therapy was effective in decreasing lipid peroxidation, suggesting a possible reduction in oxygen-derived free radical activity in traumatized skeletal muscle.

Further work is required to determine whether oxygen free radicals are primary or secondary to the damage process.

Also, the source of free radicals following injury to skeletal muscle has not yet been identified. Several potential sources of oxygen free radicals associated with skeletal muscle in exercise, trauma, and cold treatment may include: (a) reactions involved in electron transfer in the mitochondrial respiratory chain (Boveris, 1973; Sexton, 1990); (b) enzymes which catalyze oxidation reactions such as xanthine oxidase (Korthius, 1985); (c) oxidases associated with polymorphonuclear leukocytes (Fantone, 1985). Future work using this protocol should examine the extent of the effect of an exercise protocol to determine if exercise training makes skeletal muscle more resistant to oxygen free radical-derived injury, and if possibly, that long-term recovery of skeletal muscle tissue is enhanced by exercise training.

## 6.2 Conclusions

The following conclusions can be drawn from this thesis:

a. Endurance trained muscle showed an increase in citrate synthase activity.

b. Endurance exercise had no effect on indicators of oxygen free radicals in the medial gastrocnemius or soleus muscle.

c. Acute blunt trauma had no effect in indicators of oxygen free radicals on medial gastrocnemius or soleus muscles,

72 hours post trauma.

d. The administration of cold decreased indices of lipid peroxidation in the medial gastrocnemius muscle (which received the more direct effect from the cold pack). Cold treatment had no effect in indicators of oxygen free radicals on the soleus muscle.

e. There were variable results in the different treatment groups with the different muscle groups studied.

Therefore, the three hypotheses tested in the study were:

1. There was a significant difference in the citrate synthase content between trained skeletal muscle and non-trained or sedentary skeletal muscle, therefore hypothesis 1 was rejected.
2. There was a significant difference in indicators of oxygen free radical production in lipofuscin content between the treatment groups and the control group in the medial gastrocnemius muscle, therefore hypothesis 2 was rejected.
3. There was no significant difference in indicators of oxygen free radical production (whether measured by MDA or lipofuscin between treatment groups and the control group in the soleus muscle, therefore hypothesis 3 was accepted.

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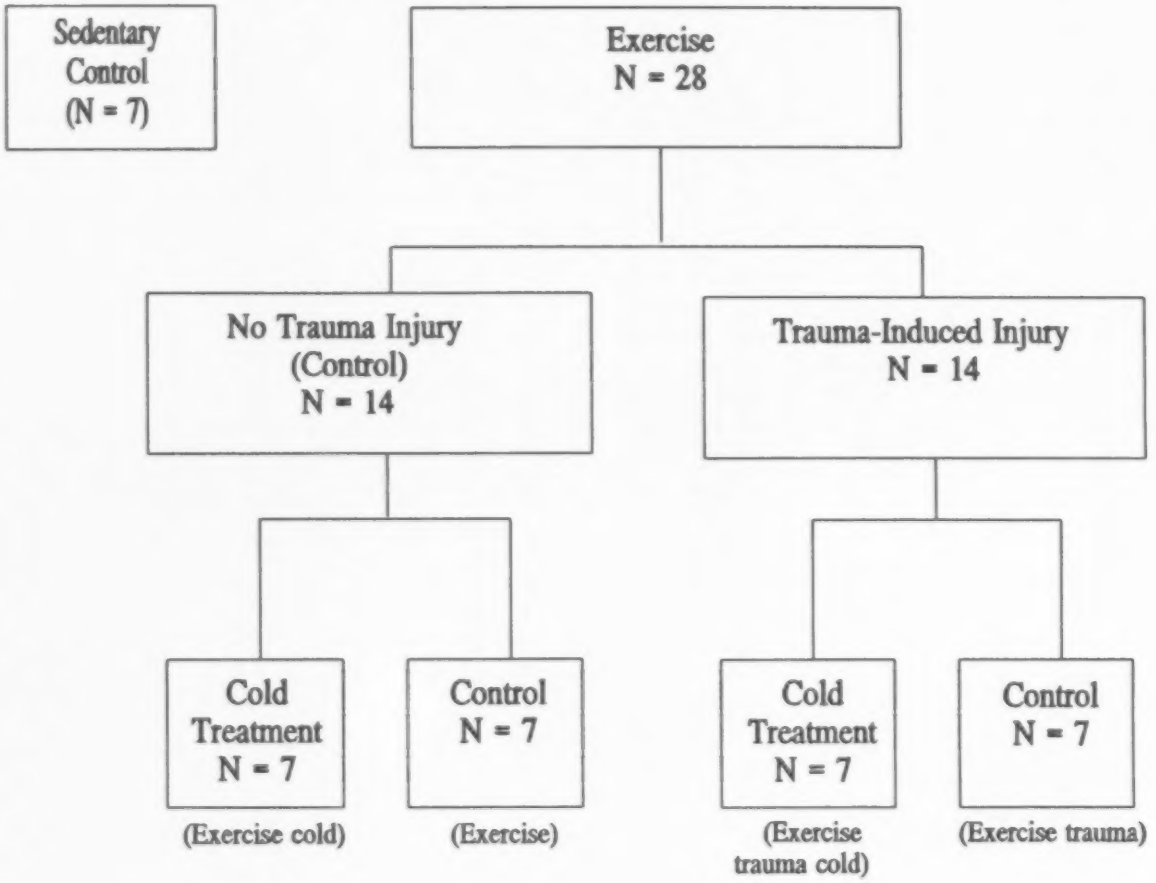
# APPENDIX A

# Figure 2: Experimental Design



# APPENDIX B

## Pilot Study

In a pilot experiment, rats were traumatized as described under Methodology (Chapter 3). Figure 3 outlines to experimental design.

In order to asses the effects of injury on indicators of oxygen free radical levels, medial gastrocnemius and soleus muscles were dissected from a healthy control, as well as from both legs of the injured rats. Trauma was delivered to the right hindlimb in each treatment group. These observations were carried out immediately post-trauma, 5 minutes, 10 minutes, 20 minutes, 30 minutes and 60 minutes post-trauma, and the muscles were analyzed for MDA and lipofuscin content.

The effects of trauma on indicators of oxygen free radical levels of medial gastrocnemius and soleus muscles were determined in the following table.

Table 7: Effects of Trauma on Indicators of Oxygen Free Radicals in skeletal muscle

| Time | Muscle | MDA umoles/mg | Lipofuscin nmoles/mg |
|---|---|---|---|
| Control | L Sol | .227 | .0182 |
| | L MG | .123 | .0206 |
| | R Sol | .269 | - |
| | R MG | .089 | .0213 |

| | | | |
|---|---|---|---|
| Immediately Post-trauma | L Sol | .197 | .0272 |
| | L MG | .127 | .0141 |
| | R Sol | .419 | - |
| | R MG | 1.250 | .3791 |
| 5 Minutes Post-trauma | L Sol | .183 | .0262 |
| | L MG | .144 | .0156 |
| | R Sol | .154 | .0317 |
| | R MG | .134 | .0163 |
| 10 Minutes Post-trauma | L Sol | .204 | - |
| | L MG | .132 | .0243 |
| | R Sol | .226 | .0328 |
| | R MG | .154 | .0221 |
| 20 Minutes Post-trauma | L Sol | .183 | .0332 |
| | L MG | .138 | .017 |
| | R Sol | .174 | .0302 |
| | R MG | .117 | .017 |
| 30 Minutes Post-trauma | L Sol | .167 | .0301 |
| | L MG | .096 | .0156 |
| | R Sol | .153 | .0372 |
| | R MG | .090 | .0163 |
| 60 Minutes Post-trauma | L Sol | .134 | .0197 |
| | L MG | .111 | .0127 |
| | R Sol | .169 | - |
| | R MG | .115 | .0109 |

* R = traumatized right hindlimb
L = non-traumatized left contralateral hindlimb

Sol = soleus muscle
MG = medial gastrocnemius muscle

In the treatment groups, trauma had no significant effect on indicators of oxygen free radical activity on medial gastrocnemius and soleus muscles in MDA and lipofuscin content

post-injury. However, trauma increased the oxygen free radical level of both muscles in the injured limb immediately post-injury, but had no significant effect on its contralateral limb.

The results from this study were inconclusive due to the small number in each group, however, indicators of oxygen free radical activity were identified, and there was a significant increase in oxygen free radical production in the muscles immediately post-trauma when compared to non-traumatized muscle, and when compared to the muscles analyzed at the specified times post-injury.

# Figure 3

## Experimental Design: Pilot Study

N = 7